JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1997, p. 2401–2404 Vol. 35, No. 9
0095-1137/97/$04.00+0

A Molecular Scheme Based on 23S rRNA Gene Polymorphisms for Rapid Identification of *Campylobacter* and *Arcobacter* Species

ANA HURTADO AND ROBERT J. OWEN*

Laboratory of Enteric Pathogens, Central Public Health Laboratory, London NW9 5HT, United Kingdom

Received 12 November 1996/Returned for modification 27 January 1997/Accepted 18 June 1997

23S rRNA gene PCR amplicons from 118 strains representing 15 species of *Campylobacter* and four species of *Arcobacter* were consecutively digested with *Hpa*II, *Cfo*I, and *Hin*fI. A reproducible and discriminatory identification scheme based on combined restriction patterns was developed by using reference strains and was applied to the identification of a variety of isolates.

Campylobacter and *Arcobacter* are classified with *Helicobacter* in the epsilon subdivision of the *Proteobacteria* (11). Identification of species within those genera by conventional biochemical tests is problematic because of generally low metabolic activity and atypical reactions of some strains. We have developed a novel but simple genomic identification method targeted at the 23S rRNA genes, which are conserved and universally distributed (16). They offer potentially greater discrimination than 16S rRNA genes because they are larger and contain more variable residues (7).

An internal region of the 23S rRNA gene was amplified from 118 strains representing 15 species of *Campylobacter* and four species of *Arcobacter* (Table 1) using primers LS1 and LS2 (4). Each strain yielded one to three amplicons of 2.6 to 3.0 kbp. Size variation was attributed to the 23S rRNA gene containing intervening sequences (IVSs) in all or some of the three copies (15). The sizes (from ~110 to ~360 bp) and locations (helices 25 and 45) of IVSs (Table 1) were determined as previously described (3).

Amplicons were consecutively digested with four restriction endonucleases (*Hae*III, *Cfo*I, *Hpa*II and *Hin*fI) chosen to yield fragments of resolvable size and number (4). Results are summarized in Table 1, and examples of patterns are shown in Fig. 1. *Hae*III digestion produced highly conserved patterns among species (data not shown). *Hpa*II generated 13 different patterns (Fig. 1A and D) and separated *Campylobacter jejuni* from *Campylobacter coli*. Six other *Campylobacter* species and two *Arcobacter* species each had species-specific patterns. Twelve *Cfo*I patterns were generated for the 15 species of *Campylobacter* (Fig. 1B), and of these 12, 7 were species specific. The four species of *Arcobacter* had a common *Cfo*I pattern (Fig. 1D). *Hin*fI gave 11 different patterns (Fig. 1C) for campylobacters, but most species fell into one of three main groups (Table 1). *Hin*fI and *Hpa*II gave similar levels of discrimination among species of *Arcobacter* (Fig. 1D).

An identification scheme based on *Hpa*II, *Cfo*I, and *Hin*fI restriction profiles (*Hae*III lacked sufficient discrimination) was developed. Each PCR-restriction fragment length polymorphism (PCR-RFLP) pattern was numbered (Table 1), and individual species codes were generated by combining triple enzyme patterns in the order *Hpa*II-*Cfo*I-*Hin*fI. The resultant identification scheme is shown in Table 1. The key findings were as follows. *Cfo*I differentiated the biochemically similar species *Campylobacter lari* (2-5-2) and *C. coli* (2-1-2) (Fig. 1B). However, *C. lari* had *Cfo*I and *Hin*fI patterns similar to those of *Campylobacter upsaliensis* (11-5-2) and were distinguishable with *Hpa*II (Fig. 1A). The genus type species *Campylobacter fetus* (4^{*c}-9-6^{*c}) and *Campylobacter hyointestinalis* (4-6-6) had similar *Hpa*II and *Hin*fI profiles but had unique species-specific *Cfo*I patterns (Fig. 1B) consistent with phenotypic identification. *Campylobacter helveticus* (3^{*t}-4*-5^{*t}) was unusual in having an *Hpa*II pattern similar to that of *Arcobacter cryaerophilus* and *Arcobacter skirrowii* (3-13-10a), although they could be identified to species level with the other two enzymes.

Despite intraspecific diversity among the 28 strains of *C. coli* (12), our technique grouped them with species-specific profiles for both *Hpa*II and *Hin*fI (2-1-2 or 2^{*e}-1^{*e}-2^{*e} with IVSs). One strain (C844/93) unexpectedly had a pattern (1^{*e}-1^{*e}-$1a^{*e}$) resembling that of *C. jejuni* yet was identified as *C. coli* by species-specific PCR (5). Thirty-four strains of *Campylobacter jejuni* subsp. *jejuni* (9), including hippurate-negative isolates (8), had a species-specific profile (1-1-1 or 1^{*e}-1^{*e}-$1a^{*e}$ with IVSs) and were different from *C. coli* in *Hpa*II and *Hin*fI profiles (Fig. 1A and C). By contrast, their differentiation by the hippurate hydrolysis test (10) was less accurate (90% positive) (14). The close relatedness between *Campylobacter jejuni* subsp. *doylei* and *C. jejuni* subsp. *jejuni* (13) were reflected in only a minor difference in *Hin*fI patterns (Fig. 1C).

*Hpa*II and *Hin*fI (Fig. 1A and C) separated *C. jejuni* and *Campylobacter hyoilei* but not *C. coli* and *C. hyoilei*, which appeared to be closely related. Biochemical tests (nitrate reduction and H_2S production) must be used in combination with the PCR-RFLP scheme to identify those species (1). However, several other species difficult to accurately identify because of a lack of suitable phenotypic tests (*C. helveticus* and *C. upsaliensis*; *Campylobacter concisus* and *Campylobacter mucosalis*) could be readily distinguished with our scheme (Table 1).

Strains of *Campylobacter sputorum* had common profiles and could be identified readily at the species level, but no RFLPs correlated with subspecies or biovar status.

A. cryaerophilus (type species) and *A. skirrowii* had identical patterns with all four enzymes (Fig. 1D), which agreed with phylogenetic analysis (2). However, *Arcobacter butzleri* and *Arcobacter nitrofigilis* had unique species-specific combinations of patterns (Table 1; Fig. 1D).

Twenty-one field strains difficult to identify by phenotypic tests were analyzed to assess our scheme, and 17 of these (86%) were readily identified. One isolate was identified as either *A. cryaerophilus* or *A. skirrowii* (the only two species not

* Corresponding author. Mailing address: Helicobacter Reference Unit, Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 44 181 200 4400, ext. 3740. Fax: 44 181 200 7874.

TABLE 1. Identification scheme for *Campylobacter* and *Arcobacter* species, based on the combination of PCR-RFLP patterns obtained with *Hpa*II, *Cfo*I and *Hin*fI

Organism	Pattern[a] obtained with:			No. of strains with[b]:	
	*Hpa*II (P patterns)	*Cfo*I (C patterns)	*Hin*fI (H patterns)	No IVS	IVS (type)
C. jejuni subsp. *jejuni*[c]	**1**	1	**1**	T + 26	
	1*[e]	1*[e]	**1a*[e]**		6 (Ile)
C. jejuni subsp. *doylei*	**1*[d]**	1*[d]	**1b*[d]**		T + 6 (IId)
	1*[g]	1*[g]	**1b*[g]**		5 (IIg)
C. coli[d]	2	1	2	T + 13	
	2*[e]	1*[e]; 3a*(1)	2*[e]		13 (Ile)
C. hyoilei	2*[e]	1*[e′]	2*[e]		T + 1 (Ile)
C. lari	2	5	2	T + 2	
C. helveticus	3*[a]	1*[a]	2*[a]		1 (IIa)
	3*[t]	4*(4); 3b*(1)	5*[t](4); 2*[t](1)		T + 4 (IIa,h,i)
C. hyointestinalis	4	**6**	6a; 6b	T + 1	
C. fetus subsp. *fetus*	4*[c]	**9**	6*[c]		T + 1 (IIc)
C. fetus subsp. *venerealis*	4*[c]	**9**	6*[c]		T + 1 (IIc)
C. concisus	5	**8a**(2); **8b**(1)	4a(2); 4b(1)	T + 2	
C. showae	5	**10**	6d	T	
C. curvus	**6**	7	**3**		T (IIb)
C. gracilis	**7**	**12**	**9**		T (IA,C; IIi,j)
C. rectus	**8**	**11**	**7**		T (ID; IIh)
C. mucosalis	**9**	7	6c	T + 3	
C. sputorum subsp. *sputorum*	**10a**	**2**	**8**	T	
	10b	**2**	**8**	1	
C. sputorum bv. *faecalis*	**10a**	**2**	**8**	1	
	10a*	**2b***	**8b***		1 (IB)
C. sputorum subsp. *bubulus*	**10a***	**2b***	**8b***		1 (IB)
	10c*	**2c***	**8c***		T (IB,-)
C. upsaliensis	**11**	5	2	2	
	11*[g]	5*[g]	2*[g]		T + 2 (IIg)
	11*[f] + 11	5*[f] + 5	2*[f] + 2		1 (IIf,-)
A. butzleri	**12**	13	10b	T	
A. nitrofigilis	**13**	13	11	T	
A. cryaerophilus	3	13	10a	T	
A. skirrowii	3	13	10a	T	

[a] Each PCR-RFLP pattern was given a number; subtypes are indicated by a lowercase letter next to the number. The presence of IVSs within the 23S rRNA is indicated by an asterisk, and subtypes due to the presence of the IVSs are distinguished by a superscript letter, as follows: a through i, type of IVS in helix 45, according to size; t, three different-sized IVSs, one in each copy of the genome. Patterns in boldface are species specific. Numbers in parentheses are the numbers of strains with a particular pattern when intraspecific variation was found.

[b] I and II, IVSs located in helices 25 and 45, respectively. A through D, type of IVS in helix 25, according to size; -, IVS not present in one of the copies of the 23S rRNA gene. T, type strain.

[c] One *C. jejuni* strain, C736/93, had a smaller IVS (about 40 bp), and therefore the patterns were slightly different and are not shown here.

[d] One *C. coli* strain, C844/93, had the patterns 1*[e]-1*[e]-1a*[e] (not shown here).

distinguished by the method), and a further three isolates shared patterns but did not match any current reference pattern. *Hpa*II patterns corresponded to either *C. coli*, *C. lari*, or *C. hyoilei*, but the *Cfo*I and *Hin*fI patterns were unique. Phenotypic tests also failed to identify the isolates to species level, although they were confirmed as *Campylobacter* by genus-specific PCR (6).

Our 23S rRNA gene-based PCR-RFLP identification scheme generated conserved restriction profiles for the species tested. The sporadic presence of IVSs increased its complexity, but it was crucial that they be accounted for when patterns were interpreted. Enlarged or multiple-band amplicons generated with primers LS1 and LS2 indicated their presence. Use of the method offers several practical advantages: (i) it is more discriminatory than phenotypic tests; (ii) it is faster and simpler than non-PCR-based molecular techniques such as DNA

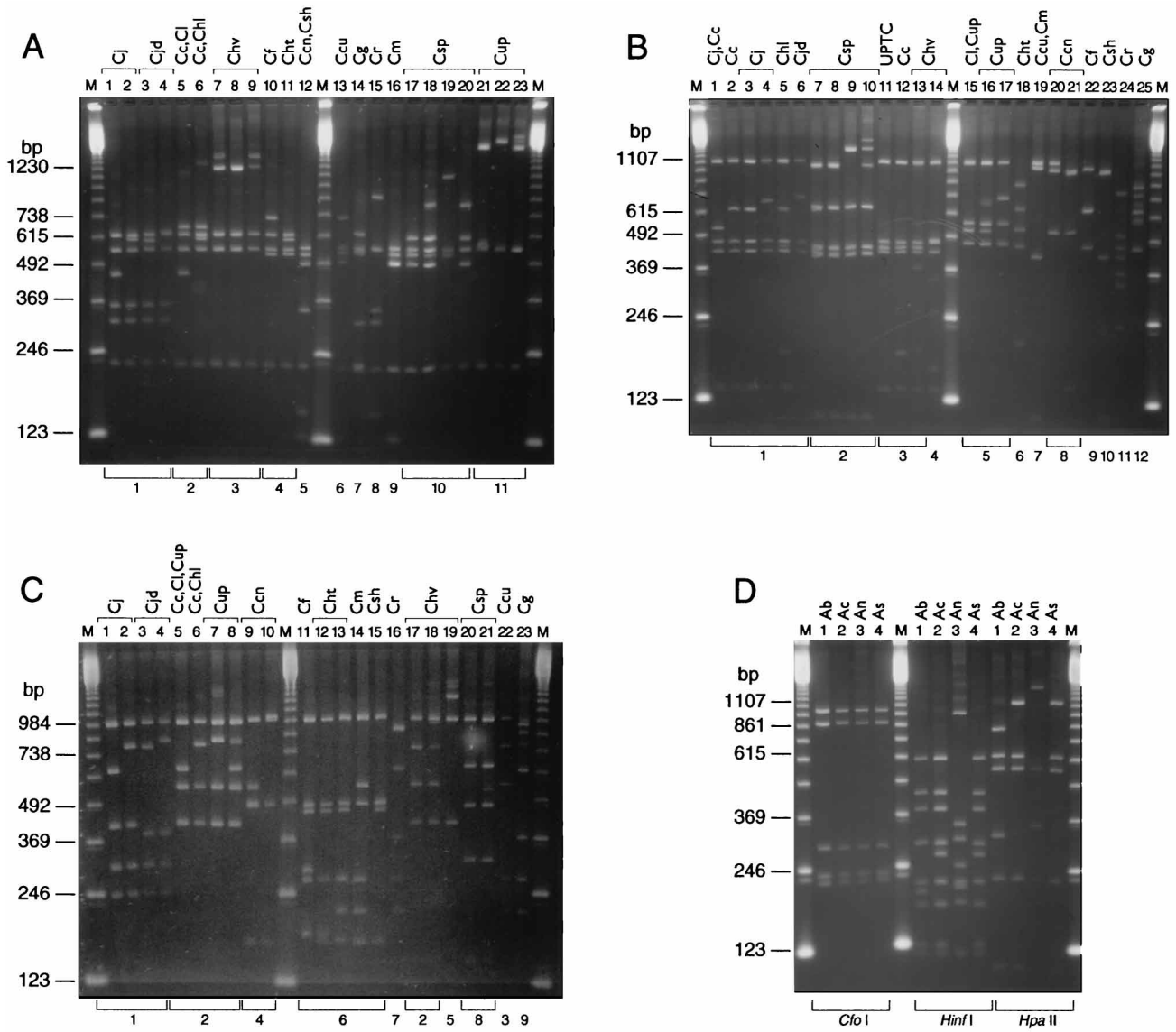

FIG. 1. Agarose gel electrophoresis of the restriction products obtained after digestion of the 23S rRNA gene amplicons with the three enzymes. (A through C) *Campylobacter Hpa*II, *Cfo*I, and *Hin*fI patterns, respectively. (D) *Arcobacter* restriction patterns (*Cfo*I, *Hin*fI, and *Hpa*II). Abbreviations: Cj, *C. jejuni* subsp. *jejuni*; Cjd, *C. jejuni* subsp. *doylei*; Cc, *C. coli*; Cl, *C. lari*; Chl, *C. hyoilei*; Chv, *C. helveticus*; Cf, *C. fetus*; Cht, *C. hyointestinalis*; Ccn, *C. concisus*; Csh, *C. showae*; Ccu, *C. curvus*; Cg, *C. gracilis*; Cr, *C. rectus*; Cm, *C. mucosalis*; Csp, *C. sputorum*; Cup, *C. upsaliensis*; Ab, *A. butzleri*; Ac, *A. cryaerophilus*; An, *A. nitrofigilis*; As, *A. skirrowii*. The pattern numbers are given at the bottom of each panel.

probe hybridization; and (iii) it is more cost effective (a single PCR) than methods requiring multiple species-specific PCRs. In conclusion, we propose that use of rRNA genes as targets for PCR-RFLP provides a stable molecular profile. The 23S rRNA gene contains more recognition sites and so improves specificity and discriminatory power. In practice, we recommend that the scheme be used in conjunction with relevant conventional phenotypic tests to avoid ambiguities. As the scheme is flexible, it can be expanded to include new *Campylobacter* and *Arcobacter* species in the future.

A.H. was the recipient of a training fellowship provided under the biotechnology program of the Commission of the European Community (BIO2-CT-94-6007).

We thank Jeremy Hardie for helpful comments.

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